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Appendix B

Antitumor Activity of 1 M Tegafur–0.4 M 5-Chloro-2,4-dihydroxypyridine–1 M Potassium Oxonate (S-1) against Human Colon Carcinoma Orthotopically Implanted into Nude Rats

Tetsuhiko Shirasaka, Koushi Nakano, Teiji Takechi,[1] Hiroyasu Satake, Junji Uchida, Akio Fujioka, Hitoshi Saito, Hiroyuki Okabe, Kiyomi Oyama, Setsuo Takeda, Norio Unemi, and Masakazu Fukushima

Institute for Pathogenic Biochemistry in Medicine [T. S.] and Cancer Research Laboratory [M. F.], Taiho Pharmaceutical Co., Ltd., 1-27 Misugidai, Hanno-City, Saitama 357, and Anticancer and Antimicrobials Research Laboratory [K. N., T. T., H. Sat., J. U., A. F., H. Sai., H. O., S. T., N. U.] and Pharmacokinetics Research Laboratory [K. O.], Taiho Pharmaceutical Co., Ltd., 224-2, Ebisuno, Hiraishi, Kawauchi-cho, Tokushima 771-01, Japan

ABSTRACT

The purpose of this study was to establish a nude rat orthotopic (organ-specific) human colorectal cancer model as an *in vivo* secondary screen for general evaluation of new anticancer agents against colorectal cancer and to evaluate practically the antitumor activity of 1 M tegafur–0.4 M 5-chloro-2,4-dihydroxypyridine–1 M potassium oxonate (S-1), a new p.o. fluoropyrimidine, in comparison to 1 M tegafur-4 M uracil [(UFT) effective on colorectal tumor in clinical]. After implantation of KM12C, a human colorectal cancer cell line, into the subserosal layer of the colon as a single-cell suspension, extensive local tumor growth and invasion to both the mucosal and the serosal sides were observed in all rats. Metastatic foci were also formed in both lymph nodes and lungs following local tumor growth in all of them. Using this method, an equitoxic dose of S-1 (15 mg/kg/day) and UFT (30 mg/kg/day) was administered p.o. for 14 consecutive days from 7 days after tumor cell implantation. S-1 showed a higher tumor growth inhibition than UFT did [S-1, 57% (significantly different from the tumor weight of the untreated group at $P < 0.05$) and UFT, 18% ($P > 0.05$)]. When both drugs were administered to nude rats bearing KM12C injected into the cecal wall for 28 consecutive days at equitoxic doses, the mean survival in the S-1 group was 16 days longer than that in the untreated group ($P < 0.01$) but that in the UFT group was only 8 days longer ($P > 0.05$). After the administration of an equitoxic dose of both drugs, S-1 gave the higher levels than UFT in various pharmacokinetic parameters as follows: area under the curve 0–24 h of 5-fluorouracil in plasma (3.5-fold), area under the curve 0–24 h of 5-fluorouracil incorporated into RNA in the tumor (1.3-fold), and thymidylate synthase inhibition rate (percentage) in the tumor (about 20%). Collectively, these findings suggested that this orthotopic human colorectal tumor model in nude rats is useful to evaluate the clinical therapeutic efficacy of drugs or therapies for colorectal cancer, and that S-1 had a higher therapeutic effect on human colorectal tumor than UFT did.

INTRODUCTION

Fluoropyrimidines have been widely used clinically in the treatment of solid tumors (1–3) since 5-FU[2] was first synthesized in 1957 (4). Because of both a good antitumor efficacy of 5-FU on gastrointestinal tract tumors as a single agent and a high incidence of gastrointestinal tract cancer cases in Japan, many attempts have been made to develop new superior 5-FU derivatives to the existing fluoropyrimidines. However, sufficient therapeutic efficacy of such drugs has not been obtained yet.

More recently we developed S-1, a new antitumor agent based on biochemical modulation of 5-FU, consisting of FT, CDHP, and Oxo in a molar ratio of 1:0.4:1 (Fig. 1). FT, which is a prodrug of 5-FU, plays a role as an effector. Both CDHP and Oxo which do not have antitumor activity themselves play roles as modulators. CDHP competitively inhibits dihydropyrimidine dehydrogenase (EC 1.3.1.2), which degrades 5-FU, about 180 times more effectively than uracil *in vitro* (5), leading to the retention of a prolonged concentration of 5-FU in blood (6). Oxo competitively inhibits pyrimidine phosphoribosyltransferase (EC 2.4.2.10), which converts 5-FU to 5-fluorouridine 5′-monophosphate *in vitro* (7). Oxo is mainly distributed in the gastrointestinal tract after p.o. administration to rats, leading to relief of gastrointestinal toxicity induced by 5-FU (7). S-1 showed a better therapeutic effect on various rats tumors and human xenografts than other p.o. fluoropyrimidines (6).

Most human tumor xenograft studies, including colorectal tumors, for the evaluation of antitumor effects of drugs utilized s.c. implantation systems for reasons of convenience and an allowance of direct quantitation of growth and therapeutic effects (8–10). However, those models have limitations for the study of the interaction of tumor cells with their relevant organ environment or organ distribution of drugs.

Recently, there has been increased interest in the use of *in vivo* models for the propagation of human tumors at organ-specific (orthotopic) sites in athymic nude mice, including renal cell carcinoma (11), colorectal carcinomas (12–15), lung carcinomas (16, 17), prostate cancer (18), breast cancer (19), and pancreatic carcinomas (20). According to the "seed and soil" hypothesis proposed by Paget (21), orthotopic implantation of tumor cells is essential for optimal growth and progression of tumors *in vivo*. However, whether such orthotopic human colorectal tumor models apply to the evaluation of new anticancer agents remains unknown.

In the present study, the orthotopic human colorectal tumor model in athymic nude rats has been used to clarify the antitumor effect of S-1 in comparison to that of UFT, which is composed of FT and uracil in a molar ratio of 1:4 and widely used as a p.o. antitumor agent in Japan (22–25). In addition, 5-FU levels in plasma, 5-FU levels incorporated into the RNA fraction (F-RNA), and TSIR in both tumor and intestinal tissue were examined to determine the mode of action of CDHP and Oxo.

MATERIALS AND METHODS

Preparation of Drugs. FT, CDHP, and Oxo were synthesized in the Taiho Pharmaceutical Co. (Tokyo, Japan). Uracil was purchased from the Yamasa Co. (Chiba, Japan). [6-^{3}H]FdUMP (551 GBq/mmol) was enzymatically synthesized from [6-^{3}H]5-fluorodeoxyuridine (New England Nuclear, Boston, MA). All other chemicals used were the highest grade of standard commercial products. S-1 was prepared by mixing with FT, CDHP, and Oxo in a molar ratio of 1:0.4:1. UFT was prepared by mixing with FT and uracil in a molar ratio of 1:4. S-1 was dissolved in a 0.5% (w/v) HPMC solution. UFT was suspended in a 0.5% HPMC solution because of the insolubility of uracil. Since the active component in both S-1 and UFT is FT, the amount of only FT

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[1] To whom requests for reprints should be addressed, at Cancer Research Laboratory, Hanno Research Center, Taiho Pharmaceutical Co., Ltd., 1-27, Misugidai, Hanno-city, Saitama 357, Japan.

[2] The abbreviations used are: 5-FU, 5-fluorouracil; S-1, 1 M tegafur-0.4 M 5-chloro-2,4-dihydroxypyridine-1 M potassium oxonate; UFT, 1 M tegafur-4 M uracil; F-RNA, 5-FU incorporated into RNA; TS, thymidylate synthase; TSIR, TS inhibition rate; FT, tegafur; CDHP, 5-chloro-2,4-dihydroxypyridine; Oxo, potassium oxonate; FdUMP, 5-fluorodeoxyuridine 5′-monophosphate; HPMC, hydroxypropylmethylcellulose; BWS, body weight suppression; TS_t, TS total; TS_f, TS free.

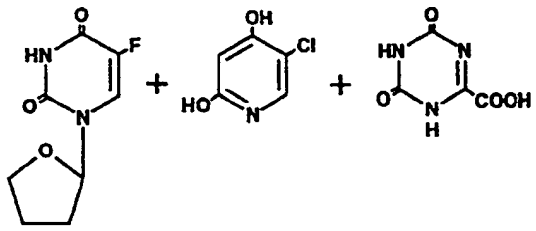

Fig. 1. Chemical structure of S-1.

in both drugs was mentioned as their dosage. The dose of each drug for *in vivo* treatment was set to the equitoxic level, *i.e.*, S-1, 15 mg/kg/day, and UFT, 30 mg/kg/day, in a volume of 0.1 ml/10 g and was administered once daily.

Animals. Female F344/N-nu rats were purchased from CLEA Japan, Inc. (Tokyo, Japan) and were free of known pathogens at the time of study. All procedures for *in vivo* experiments were performed in a specific pathogen-free barrier in our laboratory.

Tumor Cell Line and Culture. The human colorectal adenocarcinoma line KM12C (26) was provided by Dr. K. Morikawa (Iwamizawa Worker's Compensation Hospital, Hokkaido, Japan). Tumor cells were maintained in Eagle's MEM (ICN Biomedicals, Inc., Cleveland, OH) supplemented with 10% fetal bovine serum (Cell Culture Technology, Toronto, Ontario, Canada), 0.1 mM sodium pyruvate, 1% nonessential amino acids, 2 mM L-glutamine, and a 2% vitamin solution. Tumor cells were harvested from subconfluent cultures by trypsinization, washed once in serum-free medium, and resuspended in 0.9% NaCl solution for injection. Only single-cell suspensions with greater than 95% viability (tested by trypan blue exclusion) were used for *in vivo* injection.

Intracolonic Implantation of Tumor Cells and Inhibition of Tumor Growth by Drugs. The technique performed was based on the method of Morikawa *et al.* (27). Namely, nude rats were anesthetized with diethyl ether, and the abdomen was prepared for sterile surgery. The colon was exteriorized, and KM12C cells (1×10^6/0.025 ml) were injected into the colonic wall located at about 5 cm above the anus from the serosal side with a 30-gauge needle. The colon was returned to the abdominal cavity, and the wound was closed with metal clips. All rats survived after surgery. Rats were randomized into treatment or control groups (10 rats) based on body weight on 7 days after tumor cell injection. The drugs were administered p.o. for 14 consecutive days, from day 7 to day 20. The control group was given a 0.5% HPMC solution only, according to the same schedule. On day 21, body weight was determined just before they were killed with diethyl ether, and the tumors were removed and weighed. Tumor growth inhibition as an antitumor effect and body weight change as host toxicity were estimated as follows: tumor growth inhibition (%) = [1 − (mean tumor weight of treated group)/(mean tumor weight of control group)] × 100 and BWS (%) = [1 − (mean body weight of treated group)/(mean body weight of control group)] × 100.

Intracecal Implantation of Tumor Cells and Elongation of the Life Span of Nude Rats Bearing Tumors by Drugs. The technique performed was similar to that described above. In short, KM12C cells (1×10^6/0.025 ml) were injected into the cecal wall directly under the subserosa at about 3 cm above the tip of the cecum exteriorized with a 30-gauge needle. All rats survived after surgery. Rats were randomized into treatment and control groups (six or seven rats) based on body weight on 7 days after tumor cell injection (on day 0). The drugs were administered p.o. for 28 consecutive days, from day 0 to day 27. Observation was continued until all rats were dead. The value for criteria of survival prolongation was calculated as follows: increase of life span (%) = [(mean survival time of treated group)/(mean survival time of control group) − 1] × 100.

Collection of Specimens. Thirty-one days after intracolonic implantation of KM12C cells in the same manner as described above, tumor-bearing rats were divided into two groups, and then S-1 and UFT were administered p.o. At 0, 2, 4, 8, and 24 h after administration, rats were killed, and blood, tumor, and tissue of the large intestine near the tumor were collected. Blood samples were collected into tubes containing heparin and centrifuged immediately to prepare plasma.

Determination of 5-FU Levels in Plasma. Plasma samples were mixed with methanol, and the coagulated protein was removed by centrifugation. 5-FU determination was based on the method of Marunaka *et al.* (28) using a gas chromatograph-mass spectrometer (Models JGS-20kp and JMS-D 300, respectively, JEOL, Tokyo, Japan). The AUC was calculated using the trapezoidal method.

Determination of F-RNA Levels in Tumor and Tract of the Large Intestine. Isolation and quantification of 5-FU incorporated into the RNA fraction were performed according to the method of Uchida *et al.* (29). In short, RNA fractions in tissues were extracted and separated according to the method of Schneider (30). For isolation of 5-FU, RNA fractions were heated to 100°C in 6 M HCl and hydrolyzed for 24 h. Finally, 5-FU was determined using a gas chromatograph-mass spectrometer as described above.

Determination of the TSIR in Tumor and Tract of the Large Intestine. TS activity was determined as [6-^{3}H]FdUMP binding sites in the 105,000 × *g* supernatant (cytosol) of tumor tissue homogenates based on the method of Spears *et al.* (31). The samples for TS_t was prepared by causing the ternary complex present in the cytosol to fully dissociate to unbound TS at pH 8.0 in a preincubation period. In the case of TS_f samples, preincubation of the dissociation process was omitted. TS_t and TS_f samples were incubated with the [6-^{3}H]FdUMP in the presence of 5,10-methylenetetrahydrofolate for 20 min at 30°C, and the radioactivity in the acid-insoluble fraction was measured with a liquid scintillation counter. The TSIR was calculated using the following equation: TSIR (%) = $(1 - TS_f/TS_t) \times 100$.

Histological Analysis of Tumor Tissue. The tumors were fixed in 10% formalin and embedded in paraffin wax. Sections were cut at 3 μm and stained with H&E.

Statistics. The significance of differences between means was assessed using Dunnett's *t* test.

RESULTS

Growth and Metastasis of KM12C after Implantation into the Colon or Cecum. Fig. 2 shows the local tumor growth and systemic metastasis after the intracolonic injection of KM12C cells. Four days after implantation, the first tumor mass within the bowel wall was confirmed by macroscopic necropsy (Fig. 2*A*), and then extensive local tumor growth and invasion both inward and outward were observed in all rats (Fig. 2, *B–D*). Metastases to mesenteric lymph nodes and lungs (Fig. 2*E*) following local tumor growth were found in all of them. Metastases to liver were not found at all. Metastatic tumors were histologically the same as the primary intracolonic tumor. The aspect of tumor growth and metastasis after the intracecal injection of KM12C cells was similar to that after the intracolonic injection (data not shown).

The mean survival period of rats after the injection of KM12C cells into either the colon or cecum was 90.7 days (SD = 22.7, $n = 3$) or 55.0 days (SD = 4.8, $n = 4$), respectively. Necropsy revealed that the causes of death in these animals were bowel obstruction or a pulmonary death due to lung involvement.

Growth Inhibitory Effect of S-1 on KM12C Implanted into the Colon. Table 1 shows the antitumor activity of S-1 on the intracolonic KM12C tumor in comparison to that of UFT. In the treatment, the equitoxic dose was administered for 14 consecutive days. In fact, each drug brought the equivalent BWS on day 14. The mean tumor weight after S-1 treatment was less than one half of that in the control group, and this difference was statistically significant ($P < 0.05$). In addition, no lymph node metastases were observed in any of the 10 rats in the S-1 group. On the other hand, UFT did not effectively suppress tumor growth.

Elongation of the Life Span of Nude Rats Bearing KM12C Implanted into the Cecum by S-1. Table 2 shows the elongation of the life span of the intracecal KM12C-bearing rats by S-1 in comparison to that by UFT. In the treatment, the equitoxic dose was administered for 28 consecutive days, when each drug brought approxi-

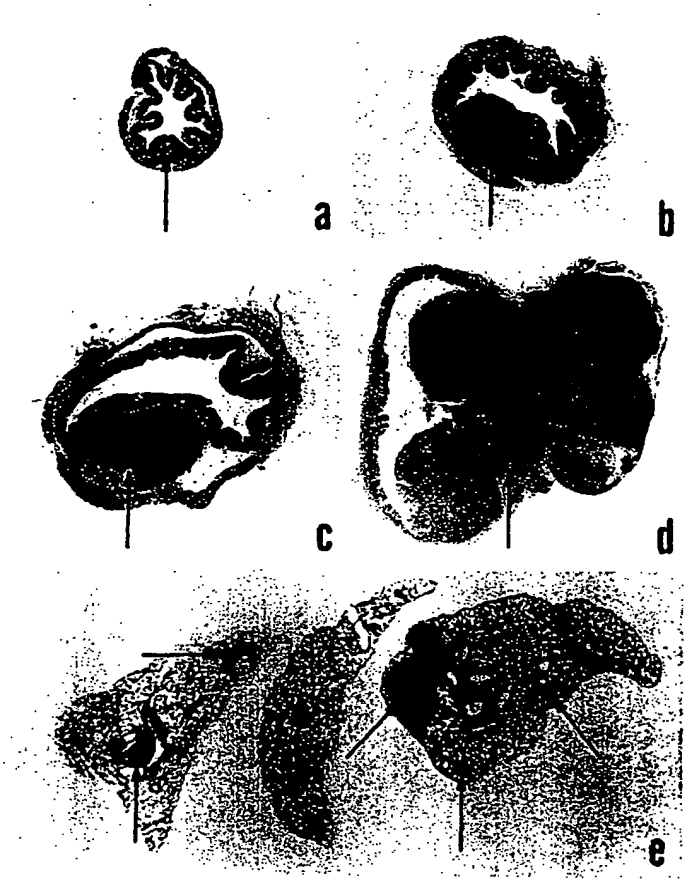

Fig. 2. Growth and metastasis of KM12C human colon adenocarcinoma in F344/N-nu rats after orthotopic implantation. Photomicrographs of a local tumor mass following intracolonic implantation with a 1×10^6 tumor cell inoculum are depicted on days 4 (*a*), 14 (*b*), 21 (*c*), and 35 (*d*). *e*, lung metastasis of KM12C in nude rats on day 42. *Arrows*, tumors.

Table 1 *Inhibitory effect of S-1 on the growth of KM12C human colon adenocarcinoma implanted into colon in F344/N-nu rats*

Group	Dose (mg/kg/day)	Body weight (g)	BWS[a] (%)	Tumor weight (g)	Tumor growth inhibition[b] (%)	Lymph node metastasis
Control		136 ± 15[c]		0.36 ± 0.27[c]		2/10
S-1	15	129 ± 6	5	0.15 ± 0.07[d]	57	0/10
UFT	30	129 ± 12	5	0.29 ± 0.23	18	1/10

[a] On day 21.
[b] On day 21.
[c] Mean ± SD.
[d] Significantly different from the control at $P < 0.05$.

Table 2 *Effect of S-1 on survival of F344/N-nu rats bearing KM12C human colon adenocarcinoma implanted into the cecum*

Group	Dose (mg/kg/day)	Body weight (g)	BWS[a] (%)	Survival period (days)	Increase of life span (%)	Metastasis[b]
Control		173 ± 13		73 ± 7		7/7
S-1	15	158 ± 10	9	89 ± 8[c]	22	6/6
UFT	30	161 ± 5	7	81 ± 10	11	7/7

[a] On day 28.
[b] Metastasis to mesenteric lymph node and lung on the day of death.
[c] Significantly different from the control at $P < 0.05$.

mately equivalent BWS. The mean survival period in the S-1 group was 16 days longer than that in the control group ($P < 0.01$), whereas that of the UFT group was only 8 days longer ($P > 0.05$).

5-FU Levels in Plasma after Administration of S-1. Fig. 3 shows 5-FU levels in plasma at various time points after the administration of the equitoxic dose of both drugs. The metabolic parameters of the S-1 group resulted in higher 5-FU levels than those of the UFT group as follows: AUC_{0-24}: S-1, 2.89 μg·h/ml; UFT, 0.83 μg·h/ml; C_{max}: S-1, 0.92 μg/ml; UFT, 0.17 μg/ml.

F-RNA Levels in Tumor and Normal Colonic Tissue after Administration of S-1. Fig. 4 shows the F-RNA levels in tumor tissue implanted into the colon at various time points after the administration of the equitoxic dose of both drugs. The metabolic parameters of the S-1 group revealed a higher F-RNA level in tumor tissue than in the UFT group as follows: AUC_{0-24}: S-1, 2.80 μg·h/g tissue; UFT, 2.11 μg·h/g tissue; C_{max}: S-1, 0.16 μg/g tissue; UFT, 0.12 μg/g tissue. Moreover, the F-RNA level in normal colonic tissue was also determined in the same animals in the S-1 group (Fig. 4). The AUC_{0-24} value of F-RNA in tumor tissue was 1.3 times as much as that in normal colonic tissue (1.58 μg·h/g tissue).

TSIR in Tumor and Normal Colonic Tissue after Administration of S-1. Fig. 5 shows the TSIR in tumor tissue implanted into the colon at various time points after the administration of the equitoxic dose of both drugs. From 4 to 24 h after drug administration, TSIR in the S-1 group was about 20% higher than that in the UFT group.

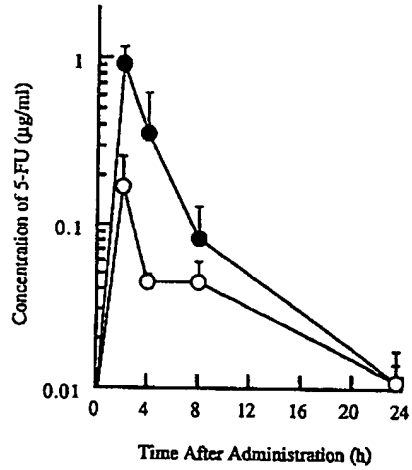

Fig. 3. 5-FU levels in plasma after p.o. administration of S-1 (15 mg/kg) or UFT (30 mg/kg) to F344/N-nu rats bearing KM12C human colon adenocarcinoma implanted orthotopically. S-1 (●) and UFT (○) were administered 31 days after implantation. At the indicated times, 5-FU levels were determined as described in "Materials and Methods." Values represent means for three rats. *Bars*, SD.

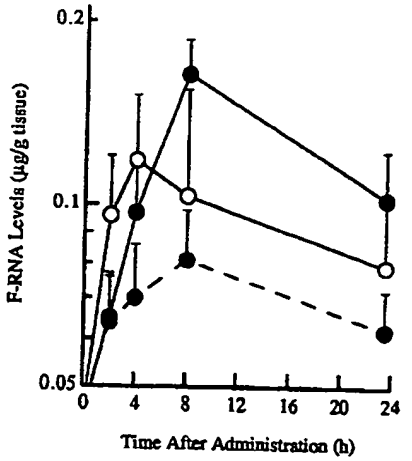

Fig. 4. F-RNA levels in tumor tissue and normal colonic tissue after p.o. administration of S-1 (15 mg/kg) or UFT (30 mg/kg) into F344/N-nu rats bearing KM12C human colon adenocarcinoma implanted orthotopically. S-1 (●) and UFT (○) were administered 31 days after implantation. At the indicated times, F-RNA levels in tumor (——) and normal (– – –) tissue were determined as described in "Materials and Methods." Values represent means for three rats. *Bars*, SD.

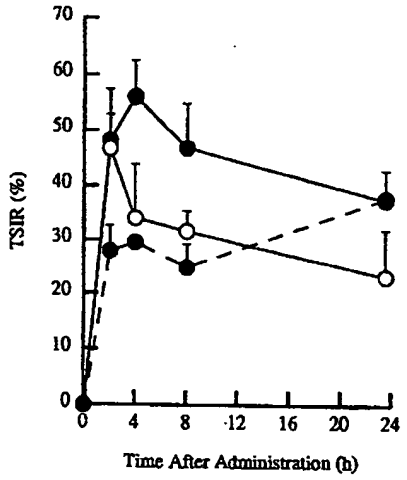

Fig. 5. TSIR in tumor tissue and normal colonic tissue after p.o. administration of S-1 (15 mg/kg) or UFT (30 mg/kg) into F344/N-nu rats bearing KM12C human colon adenocarcinoma implanted orthotopically. S-1 (●) and UFT (○) were administered 31 days after implantation. At the indicated times, TSIR in tumor (——) and normal (– – –) tissue were determined as described in "Materials and Methods." Values represent means for three rats. *Bars*, SD.

Moreover, TSIR in normal colonic tissue was also determined in the same animals in the S-1 group (Fig. 5). From 2 to 8 h, TSIR in tumor tissue was 20–36% higher than that in the normal tissue.

DISCUSSION

Paget (21) originally proposed that human tumor cell populations require organ site-specific interaction for optimal maintenance and progression. This concept has been widely supported by numerous studies (11–20) including colorectal carcinoma (12–15).

Recently, we have established the colorectal tumor models by the implantation of the human colorectal adenocarcinoma line KM12C into the colon or cecum in nude rats. Since intracolonic tumors were much closer to clinical tumors than s.c. tumors from the view of the histology of tumor growth or metastasis (Fig. 2), this system was applicable to the evaluation of the tumor growth inhibitory effect by drugs (Table 1). The preparatory experiment to confirm the survival period of rats after the injection of KM12C cells into either the colon or cecum revealed that the deviation of survival time of intracecally implanted rats (SD, 4.8 days) was smaller than that of intracolonic implanted rats (SD, 22.7 days); therefore, the intracecal implant system was used for the evaluation of the elongation of life span by drugs (Table 2). This system, however, did not seem to be applicable to the evaluation of the tumor growth inhibitory effect, since intracecal tumor growth was too fast to be suppressed by drugs (data not shown).

Liver metastases, which we failed to produce in nude rats, are the most frequent occurrence in colorectal tumor patients. Morodomi *et al.* (32) have performed orthotopic implantation in nude mice and nude rats using the human colon carcinoma KM12SM, which is a subline of KM12C (27). They found liver metastases in 0 of 11 nude rats and found them in 57% of nude mice (12/21). Where metastases are formed may be due to a combination between host species and a tumor line.

Collectively, these models have some merits: (*a*) orthotopic implantation in the nude rat was technically much easier than that in the nude mouse; (*b*) local tumor growth and metastasis to both lymph nodes and lungs were completely achieved; (*c*) tumor growth inhibition and elongation of the life span by drugs can be evaluated in a comparatively short period; and (*d*) features of tissue distribution, *i.e.*, distribution into normal tissue or tumor tissue in the intestinal tract, of the drug can be clarified. These features indicate that these orthotopic colorectal tumor models are useful in evaluating the clinical therapeutic efficacy of drugs or therapies on colorectal cancer.

Recently, a long-term continuous venous infusion of 5-FU has been used as an optimal schedule of 5-FU compared with a bolus injection of 5-FU (33–36), the prolonged retention of 5-FU in blood leading to a higher therapeutic effect. In this manner of treatment, the dose-limiting factor is not myelosuppression but gastrointestinal disorders such as diarrhea and mucositis (37, 38).

In the present study, S-1 inhibited tumor growth on KM12C implanted into the colon more than UFT at the equitoxic dose (Table 1). Moreover, S-1 showed a significant elongation of the life span of KM12C-bearing nude rats with implants in the cecum, although UFT did not (Table 2). The high antitumor activity of S-1 on orthotopic colorectal tumor was in accordance with the higher level of 5-FU in plasma (Fig. 3) and F-RNA (Fig. 4) and TSIR (Fig. 5) in the tumor. It is considered that these effects were due to CDHP, which is a 180 times more potent inhibitor of 5-FU degradation than uracil (5) to make the level of 5-FU in blood prolonged like continuous venous infusion of 5-FU (6).

It is interesting that the levels of F-RNA and TSIR in normal colonic tissue were lower than those in tumor tissue (Figs. 4 and 5). Shirasaka *et al.* (7) reported that Oxo was mainly distributed in the gastrointestinal tract after p.o. administration to Yoshida sarcoma-bearing rats, and that it inhibited the formation of 5-fluorouridine 5′-monophosphate and F-RNA from 5-FU in the small intestine and markedly reduced injury of the gastrointestinal tract and severe diarrhea without influencing the antitumor effect of UFT. These findings suggest that Oxo could selectively decrease the anabolism and the cytotoxicity of 5-FU in normal tissue of the intestine. Additional biochemical features of Oxo are under investigation in our laboratory.

Collectively, these findings indicate that CDHP and Oxo gave S-1 a higher antitumor effect than UFT on orthotopic KM12C colorectal tumors. S-1 is expected to have a high therapeutic efficacy for the treatment of colorectal tumors clinically.

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